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Interleukin 1 receptor antagonist mediates the beneficial effects of systemic interferon beta in mice: implications for rheumatoid arthritis

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ABSTRACT

Objectives Interferon beta (IFN β) therapy is effective in multiple sclerosis and murine models of arthritis. Surprisingly, systemic IFN β treatment induces only minimal improvement in rheumatoid arthritis (RA). To explain this paradox, the authors evaluated the mechanism of IFN β benefit in passive K/BxN arthritis and the effect of IFN β treatment on RA synovium.

Methods Interleukin 10 (IL-10) null, IL-1 receptor antagonist (IL-1Ra) null, IL-1Ra transgenic and wild-type mice were administered K/BxN serum and in some cases treated with IFN β or normal saline. Clinical response and histological scores were assessed. Gene expression was measured by quantitative PCR. Serum IL-1Ra and IL-6 were measured by ELISA. Paired synovial biopsy specimens from RA patients pre-IFN β and post-IFN β treatment (purified natural fibroblast IFN β (Frono) subcutaneously three times weekly 6 million IU, 12 million IU or 18 million IU) were immunostained for IL-1Ra and IL-10.

Results *Il1m* transgenic mice had an attenuated course of arthritis, whereas *Il1m*^{-/-} and *Il10*^{-/-} mice had more severe serum transfer arthritis than wild-type mice. Daily IFN β treatment significantly decreased arthritis severity in *Il10*^{-/-} but not *Il1m*^{-/-} mice. IFN β treatment did not reduce the histological scores in *Il1m*^{-/-} mice or gene expression of articular cytokines and chemokines. Paired synovial biopsy specimens from RA patients treated with IFN β demonstrated a trend towards increased IL-1Ra and reduced IL-10 expression on day 85 levels compared with pretreatment specimens.

Conclusions The anti-inflammatory effects of IFN β in passive K/BxN arthritis are dependent on IL-1Ra, but not IL-10. Systemic IFN β treatment in RA increases synovial IL-1Ra production, but also decreases IL-10 production.

Rheumatoid arthritis (RA) is an immune-mediated disease characterised by systemic and synovial inflammation, resulting in bone and cartilage destruction. Cytokine-modulating therapies have been effective in some but not all patients. In addition to approaches aimed at reducing the levels of proinflammatory cytokines, administering anti-inflammatory cytokines has been studied. Interferon β (IFN β) is such a factor with immunomodulatory effects and its expression is increased in RA synovium.¹ IFN β can inhibit the expression of proinflammatory cytokines such as interleukin 1 β (IL-1 β) and tumour necrosis factor (TNF), and increase anti-inflammatory factor production, such as IL-10 and IL-1 receptor antagonist (IL-1Ra).²⁻⁴

Despite the promising in-vitro and animal model results trials with systemic IFN therapy in RA did not demonstrate efficacy by clinical, radiographic, biochemical and histological parameters.⁵⁻⁶

We previously reported the increased sensitivity to IFN β therapy in mutant mice that had a genetic disruption in a gene associated with the proinflammatory response to type I IFN.⁷ I κ -B kinase ϵ (IKK ϵ) deficient mice had less arthritis than wild-type mice and responded to a lower dose of IFN than controls.⁷ IFN-treated mice had an increase in *Il1m* (which encodes IL-1Ra) transcripts in their paws and serum; however, there was no difference in the levels of IL-10 compared with saline-treated mice.⁷ Therefore, IL-1Ra could be implicated as the potential mediator of the beneficial effects of IFN therapy.

To test the relative contributions of IL-10 and IL-1Ra as the key mediators of the beneficial effects of IFN therapy we examined mice that had targeted mutations in these two genes. Using the K/BxN serum transfer model we examined the relative severity of arthritis induction in these two strains and their response to IFN treatment. To correlate the findings with human disease, previously banked paired pre-IFN and post-IFN synovial tissue samples from a previous trial were stained for IL-1Ra and IL-10.⁸ Overall, the results suggest that the IFN β benefit is dependent on IL-1Ra in passive K/BxN arthritis in mice, and that systemic IFN β increases synovial IL-1Ra yet also diminished IL-10 expression in RA.

METHODS

Mice

KRN T-cell receptor transgenic mice were a gift from Drs D Mathis and C Benoist (Harvard Medical School, Boston, Massachusetts, USA) and Institut de Génétique et de Biologie Moléculaire et Cellulaire (Strasbourg, France),⁹ and were maintained on a C57Bl/6 background (K/B). Arthritic mice were obtained by crossing K/B with NOD/Lt (N) animals (K/BxN). C57Bl/6, *Il1m* transgenic,¹⁰ *Il10*^{-/-},¹¹ *Il1m*^{-/-}¹² and NOD/Lt mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The mice were bred and maintained under standard conditions in the University of California, San Diego Animal Facility, which is accredited by the American Association for Accreditation of Laboratory Animal Care. All animal protocols receive previous approval by the institutional review board.

Serum transfer and arthritis scoring

Arthritic adult K/BxN mice were bled and the sera were pooled. Recipient mice were injected with 150 μ l intraperitoneally on day 0. Some groups of mice also received IFN β (Chemicon (3.6×10^7 U/mg or 1000 U=28 ng)) intraperitoneally, or normal saline (NS). Clinical arthritis scores were evaluated using a scale of 0–4 for each paw for a total score of 16. Ankle thickness was measured with a caliper (Mitutoyo, Kawasaki, Japan) in mm.¹³

Histology

Whole knee joints and hind paws were fixed in 10% formalin, decalcified, trimmed and embedded. Sections were prepared from the tissue blocks and stained with haematoxylin and eosin and Safranin O (HistoTox, Boulder, Colorado, USA). Histopathological scoring was performed as previously described on a scale of 0–4 for inflammation, erosion and cartilage damage.¹³

Gene expression

The wrists of mice were snap frozen and pulverised as a pool. Total RNA was isolated using the PerfectPure RNA Fibrous Tissue Kit (5 PRIME, Gaithersburg, Maryland, USA) and complementary DNA was prepared with the qScript cDNA SuperMix kit (Quanta, Gaithersburg, Maryland, USA). The messenger RNA levels for matrix metalloproteinase 3 (MMP3), IFN regulatory factor 7 (IRF7), C-X-C motif chemokine 10 (CXCL10)/interferon gamma-induced protein 10 (IP-10), IL-6, IFN β and IL-1Ra were quantified by real time reverse transcriptase PCR using commercially designed and prepared primer and probe sets (Applied Biosystems, Foster City, California, USA) on a Bio-Rad MiQ (Hercules, California, USA) detection system. Fold induction was calculated by $2^{-\Delta\Delta Ct}$, normalising to actin and using RNA from uninjected mice of the same genotype as the baseline comparator. Each sample was analysed in triplicate.

Cytokine measurement

The sera were assayed for IL-1Ra and IL-6 using commercial capture ELISA (R&D Systems, Minneapolis, Minnesota, USA).

Immunohistochemistry and semiquantitative scoring

Synovial biopsy specimens from a previously reported phase I trial were retroactively examined.⁸ Briefly, all patients fulfilled the American College of Rheumatology criteria for RA. Active RA was defined as six tender joints, six swollen joints and at least one of the following two criteria: duration of morning stiffness 45 min or an erythrocyte sedimentation rate of 28 mm/h. All patients gave informed consent, and the study protocol was approved by the Medical Ethics Committee of the Leiden University Medical Center. The patients were treated for 12 weeks with purified natural fibroblast IFN β (Frone; Ares-Serono, Geneva, Switzerland), which was injected subcutaneously three times a week at 6 million IU, 12 million IU or 18 million IU. Small-bore arthroscopy (2.7 mm arthroscope; Storz, Tuttlingen, Germany) was performed under local anaesthesia. Biopsies of synovial tissue were obtained from the entire joint using a 2-mm grasping forceps (Storz) before and 3 months after treatment. The tissue was collected and snap-frozen en bloc in TissueTek OCT (Miles, Elkhart, Indiana, USA) by immersion in methylbutane (-70°C). Frozen blocks were stored in liquid nitrogen until sectioned for staining. There was sufficient tissue in the blocks from five patients who had been biopsied before IFN β treatment and on day 85 of treatment for analysis. Five micrometre sections were cut in a cryostat and mounted on glass slides. The sections were immunostained with anti-IL-1Ra (R&D

Systems Europe, Abingdon, UK) and anti-IL-10 (R&D Systems Europe) and detected as previously described including biotinylated tyramine for amplification.⁸ After immunohistochemical staining, sections were scored semiquantitatively on a five-point scale by two independent observers.¹⁴ Minor differences between the observers were resolved by mutual agreement.

Statistics

Significance was assessed using area under the curve analysis, analysis of variance for multiple comparisons and the Mann–Whitney U test was used for pairwise comparisons using Prism software (version 5.0).

RESULTS

Regulation of passive K/BxN arthritis by IL-1Ra and IL-10

We previously reported that genetic deficiency in IFN α/β R accelerated arthritis in K/BxN serum transfer arthritis, and C57BL/6 mice had an attenuated clinical course when treated with IFN β .⁷ We noted that diminished arthritis in IFN β -treated mice correlated with higher serum levels of IL-1Ra and mRNA levels of sIL-1Ra in arthritic paws.⁷ Although IL-10 has been implicated as a mediator of the anti-inflammatory response to IFN β , serum levels of IL-10 in IFN β -treated mice were similar to controls. To assess further the relative contributions between IL-1Ra and IL-10 we examined the effects of the constitutive production of IL-1Ra and genetic disruption in the *Il1rn* gene (figure 1). The IL-1Ra overexpressing transgenic mice had a very mild clinical course and minimal damage after 10 days by histology. Histology scores for IL-1Ra transgenic mice averaged 0.3 ± 0.2 , 0.3 ± 0.2 and 0 ± 0 compared with 3.7 ± 0.3 , 3.7 ± 0.2 and 3.0 ± 0.0 for wild-type mice for inflammation, bone erosion and cartilage damage, respectively ($n=6$ per group, $p<0.001$). In contrast the IL-1Rn-deficient mice had more severe paw swelling than wild-type littermates ($p<0.01$) (figure 1B). IL-10 deficiency also increased arthritis severity ($p<0.01$) (figure 1C).

Il1rn^{-/-} mice are refractory to IFN β therapy whereas *Il10*^{-/-} mice are responsive

Our previous studies suggested that IL-1Ra, but not IL-10, is regulated by IFN β in passive K/BxN.⁷ Because this model is exquisitely dependent on IL-1, we hypothesised that the protective effect of IFN β is mediated by IL-1Ra. The effect of IFN β on arthritis in *Il1rn*^{-/-} mice was determined to test this possibility. Unlike wild-type mice (figure 2A) the *Il1rn*^{-/-} mice did not clinically respond to IFN β treatment (figure 2B). However, the IL-10 null mice responded to daily IFN β injections ($p<0.05$ treated compared with controls) (figure 2C). Synovial inflammation, bone erosion and cartilage damage were also unaffected by IFN β treatment in *Il1rn*^{-/-} mice. Treatment with IFN β significantly improved the histological scores in wild-type littermates ($p<0.01$) (figure 3). The IL-10^{-/-} mice treated with IFN β had a reduction in their histology score, with means of 0.4 ± 0.2 , 1 ± 0.3 and 0.4 ± 0.2 for inflammation, bone erosion and cartilage damage, respectively, compared with 1.8 ± 0.5 , 3.0 ± 0.0 and 2.0 ± 0.5 for NS-treated mice ($n=5$ /group, $p<0.05$).

Enhanced expression of proinflammatory cytokine and MMP3 transcripts in *Il1rn*^{-/-} mice

IFN β treatment reduced the levels of IL-6 and MMP3 mRNA transcripts and modestly increased the IFN response gene transcription (IRF7 and IP-10) in wild-type mice compared with NS ($p<0.01$) (figure 4). In contrast IFN β treatment did not reduce the

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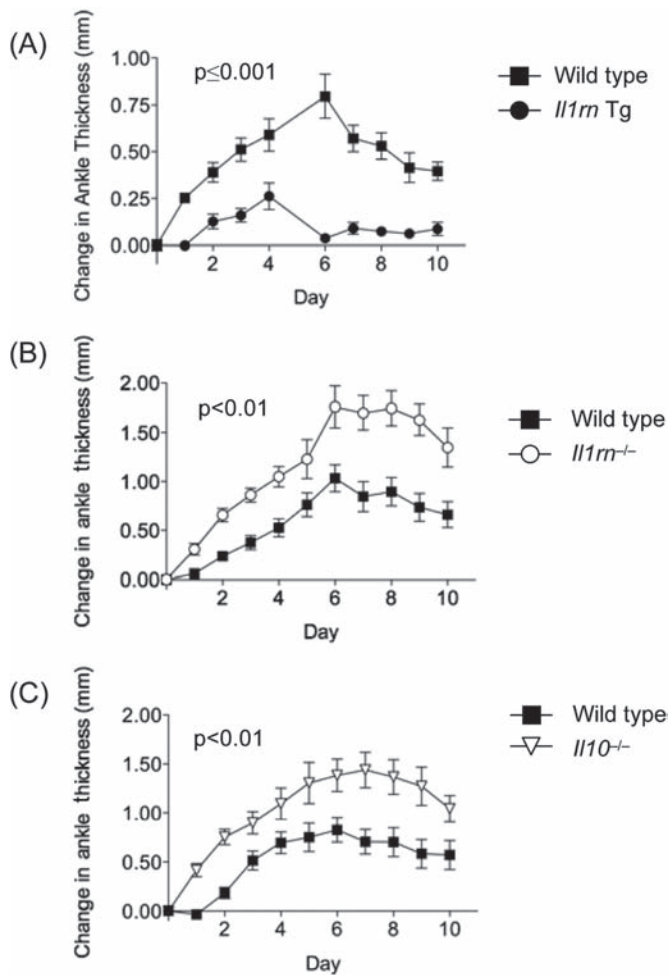


Figure 1 Genetic manipulation of *Il1rn* and *Il10* modulates arthritis. Groups of adult mice were injected with pooled K/BxN sera on day 0 and the ankle thickness was serially measured. (A) *Il1rn* transgenic mice (closed circle) have an attenuated course of arthritis compared with wild-type littermates (black square). Shown are the averages \pm SEM for 15 mice/group pooled from three experiments (p < 0.001). (B) *IL1Ra*-deficient mice (open circle) have an accentuated course of arthritis relative to wild-type littermates (black square). Shown are the averages \pm SEM for 10 mice/group pooled from three experiments (p < 0.01). (C) Interleukin 10-deficient mice (open triangle) have an accelerated course of arthritis and wild-type littermates (black square). Shown are the averages \pm SEM for nine mice/group pooled from two experiments (p < 0.01).

transcription of these genes commonly associated with inflammatory arthritis in IL-1Ra-deficient mice. The *Il1rn*^{-/-} mice treated with IFN β demonstrated a small increase in IRF7 and IP-10 mRNA transcription, indicating at least a partial biological response to the treatment. Notably, IL-1Ra null mice with arthritis also had approximately a twofold change of IL-10 mRNA expression compared with uninjected mice.

Persistently elevated IL-6 serum levels in *Il1rn*^{-/-} IFN β -treated mice

Control arthritic mice had increases in the levels of IL-6 in their sera (figure 5). The level of IL-6 was lower in IFN β -treated wild-type and *Il10*^{-/-} mice, but not *Il1rn*^{-/-} mice. Both the wild-type and the *Il10*^{-/-} mice had increases in the levels of serum IL-1Ra in response to IFN treatment. Serum levels of IL-1Ra in *Il1rn*^{-/-} mice were below the sensitivity of the assay.

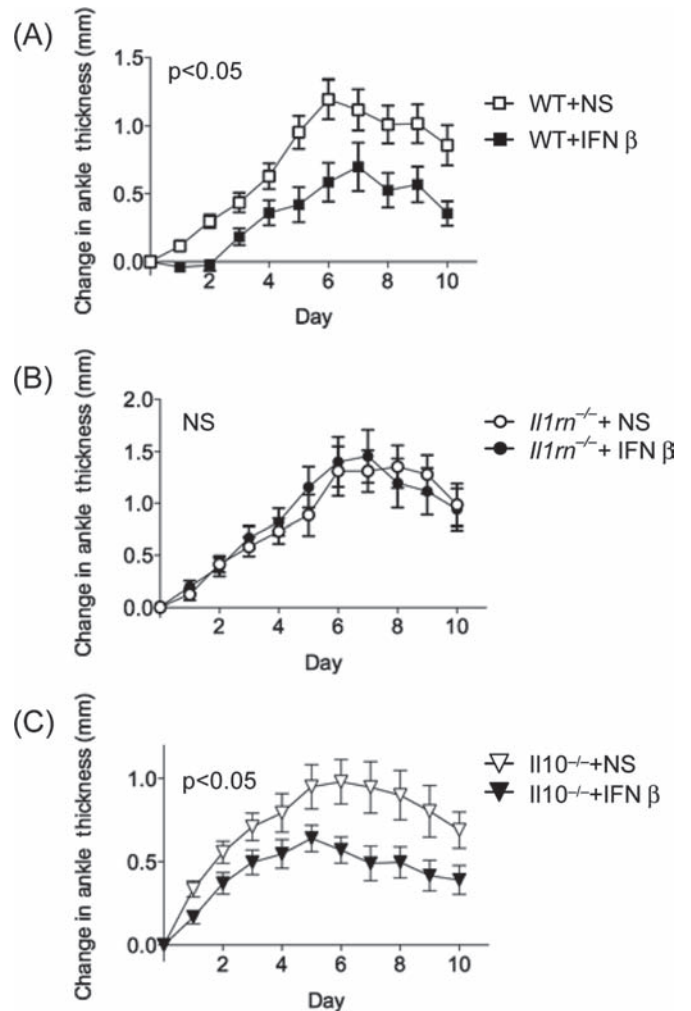


Figure 2 Interleukin 10 (IL-10) null mice are sensitive to interferon β (IFN β), but not IL-1 receptor antagonist null mice. Groups of mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera and with 1000 IU IFN β , or normal saline (NS) daily. Ankle thickness was measured daily with a caliper. Shown are the means \pm SEM from (A) 10 wild-type (WT) mice/group, (B) 13 *Il1rn* mice/group and (C) 10 *Il10*^{-/-} mice/group from two to three pooled experiments each. WT and *Il10*^{-/-} mice significantly responded to daily 1000 IU IFN β (p < 0.05), whereas the *Il1rn*^{-/-} mice did not.

Synovial samples post-IFN β treatment have increased IL-1Ra and decreased IL-10 by immunostaining

During a previous phase I trial, patients were treated for 12 weeks with purified natural fibroblast IFN β , which was injected subcutaneously three times a week.⁸ Synovial biopsies were obtained before the initiation of treatment and at 85 days post-treatment. These tissue samples were embedded, frozen and stored. There was sufficient tissue in the blocks from five of the 11 patients to compare samples from before treatment and 85 days after treatment. Sections were immunostained for IL-10 and IL-1Ra and semiquantitatively scored as previously described.¹⁴ After IFN treatment the level of IL-10 staining decreased in these patients and the level of IL-1Ra increased during the treatment interval (figure 6).

DISCUSSION

IFN β was developed as a therapeutic agent in autoimmune diseases due to its anti-inflammatory activities. Similar to other

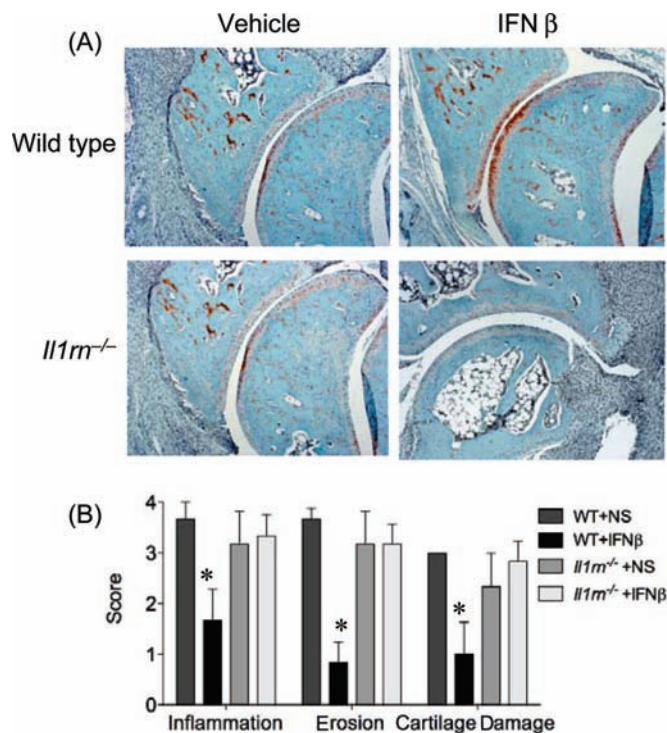


Figure 3 Persistent joint inflammation and damage in *Il1rn*^{-/-} mice despite interferon β (IFN β) treatment. (A) Wild-type (WT) and *Il1rn*^{-/-} mice were injected intraperitoneally on day 0 with 150 μ l of pooled K/BxN sera and treated with daily injections of normal saline (NS) or 1000 IU IFN β intraperitoneally. On day 10 the mice were killed, the hind limbs were removed and one ankle from each mouse was prepared for histological scoring. Representative sections with Safranin O staining are shown at 40 \times magnification. (B) Shown are the average inflammation, erosion and cartilage damage scores \pm SEM (six mice/group). * p <0.01 by analysis of variance with Bonferroni comparison.

biological therapies, this treatment has not been uniformly effective. In preclinical studies IFN β demonstrated efficacy in rodent and non-human primate models of RA.^{15 16} Daily systemic administration of IFN β reduced paw swelling, decreased serum levels of anticollagen antibodies and improved histological scores in DBA/1 mice with collagen-induced arthritis (CIA).¹⁶ Similarly, systemic treatment with recombinant IFN β in rhesus monkeys with CIA resulted in remarkable clinical improvement and decreased serum levels of C-reactive protein.¹⁵ Despite the early promise therapeutic trials in with IFN β treatment in RA had a minimal effect on synovial histology or clinical manifestations of RA.^{6 16}

Investigating the complexities of the in-vivo activities of IFN β might lead to optimising its use clinically either as a single agent or in combination with other therapeutic options. As the IFN receptors are widely expressed, the effects of IFN β might span both the adaptive and innate immune compartments by direct or indirect means. Although CIA is largely a T-cell-dependent model, an earlier report suggested that the effects of IFN β were not limited to T-cell-dependent mechanisms, but were also mediated by stromal cells and osteoclasts.¹⁷ We thus utilised the K/BxN model in these studies as this model was not B or T-cell dependent, yet still responds to IFN β treatment.^{7 9}

Previous mechanistic studies suggested the beneficial effects of IFN β treatment including a reduction in pro-inflammatory cytokines release such as IL-1 β and TNF, while enhancing IL-10 and IL-1Ra production by leucocytes and fibroblast-like synoviocytes in vitro.^{3 16 18 19} Consistent with these observations, we

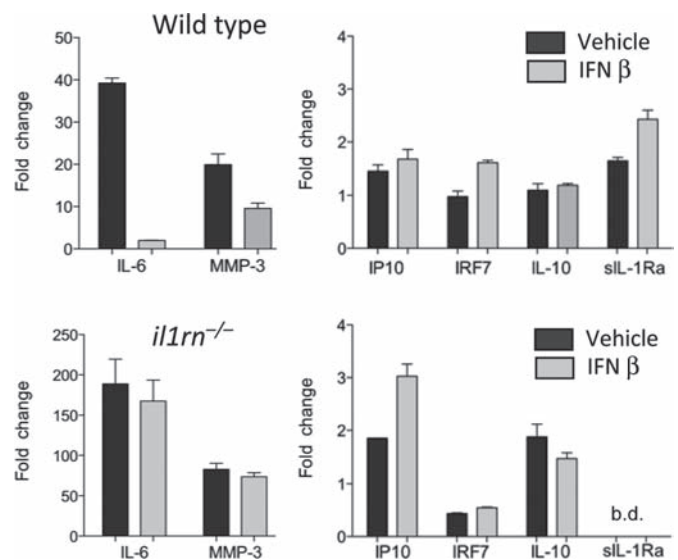


Figure 4 Gene expression profiles of interlukin 1 receptor antagonist null and wild-type arthritic joints. Three mice per group were injected on day 0 with 150 μ l of pooled K/BxN sera and then received daily injections of 1000 U of interferon β (IFN β) or normal saline. The wrist joints were removed on day 4, snap frozen and pulverised. RNA was isolated from pooled wrists using commercial kits and the relative levels of gene expression were assessed by quantitative PCR and normalised to actin. The fold induction is compared with the relative level of messenger RNA expression of uninjected control mice. Shown are the averages of replicates pooled from two experiments + SEM. The amount of *Il1rn* transcript was below detection (b.d.) in the *Il1rn*^{-/-} mice. IRF7, IFN regulatory factor 7; IL-10, interleukin 10; MMP, matrix metalloproteinase.

noted that IL-10 and IL-1Ra-deficient mice developed accelerated paw swelling compared with wild-type controls. However, the benefit of IFN β treatment in our experiments was not due to IL-10, as IL-10 null mice responded to IFN β . The IL-1Ra-deficient mice were, however, refractory to treatment. These results suggest that IL-1Ra is the predominant active mediator for improvement with IFN β treatment in this model of arthritis.

Despite the lack of efficacy in RA, IFN β has proved effective in some cases of multiple sclerosis.²⁰ The murine experimental autoimmune encephalomyelitis (EAE) model of multiple sclerosis is dependent on the production of both IL-17 and IFN γ by T helper cells for the full manifestation of disease and responds to IFN β treatment by reducing IL-17.^{21–24} The most pathogenic T cells in this murine model are those that co-express IL-17 and IFN γ .²⁵ This effect is not limited to the EAE model as IFN β also reduces IL-17 production by human T lymphocytes in vitro.^{26–28} In both humans and mice IL-1 β contributes to the priming environment that is conducive to T cells developing an IL-17-secreting phenotype (Th17).^{25 29} Notably, the K/BxN passive transfer model is T-cell and IL-17 independent and the effects on adaptive immunity as a mechanism explain our results.^{9 30}

IL-1Ra is only a modestly effective therapeutic agent in RA.³¹ Other IL-1-directed therapies, including monoclonal antibodies, soluble IL-1 receptors and caspase 1 inhibitors, also show limited benefit.^{32–35} The minimal efficacy of systemic IFN β in RA might be related to the limited role of IL-1 on the clinical manifestations of this particular disease. Alternatively, a reduction in the priming of Th17 cells due to IL-1Ra treatment might be circumvented by redundant priming mechanisms or the expression of IL-17 in synovial mast cells.³⁶ The increase in IL-1Ra expression

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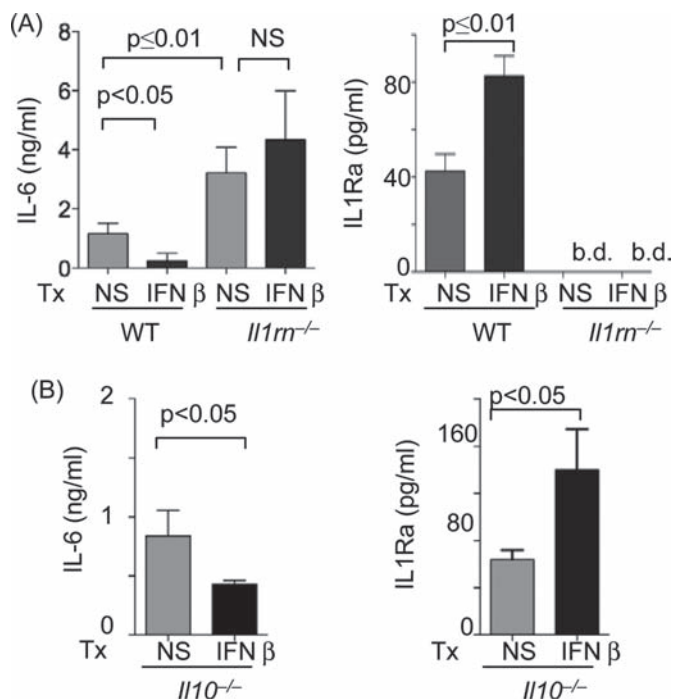


Figure 5 Interleukin 6 (IL-6) remains elevated in the sera of interferon β (IFN β) treated *Il1m*-deficient mice. Mice were injected with 150 μ l of pooled K/BxN and then treated with daily injections of normal saline (NS) or 1000 IU IFN β . The mice were bled on day 10, and the serum interleukin 1 receptor antagonist (IL-1Ra) and IL-6 amounts were measured by ELISA. (A) Shown are the means for eight to 10 wild-type (WT) or *Il1m*^{-/-} mice/group from three experiments. The level of IL-1Ra was below detection (b.d.) in the *Il1m*^{-/-} mice. (B) Shown are the averages of five *Il10*^{-/-} mice/group from one experiment. Statistical differences were assessed by the Mann-Whitney U test. Tx, treatment.

observed in RA synovium and in murine arthritis could thus explain the divergent results in the two species. IL-1 is a pivotal cytokine in passive K/BxN arthritis, and the disease is markedly attenuated by deficient IL-1R signalling.^{13–37} The increase in IL-1Ra in RA patients would not have the same impact on clinical signs and symptoms.

Reduced IL-10 in the human synovial samples after IFN β treatment was consistently observed and could also contribute to a reduction in the immunosuppressive effect of IFN β . The effect on IL-10 represents another difference between RA and passive K/BxN arthritis. Although IL-10-deficient mice had an exacerbated clinical course, they still responded to IFN β treatment.

The data on IFN β therapy in RA focus on systemic therapy. It is possible that higher concentrations or continuous delivery,³⁸ such as local gene therapy in the synovium,^{39–40} might activate additional mechanisms that possibly suppress synovial inflammation in RA.⁵ Frequent dosing might be required to sustain the activity of intracellular molecular signalling pathways responsible for regulating IFN β -induced gene expression.⁴¹ Still, IFN β at the dosage used had a biological effect, as noted by altered IL-1Ra and IL-10 expression in the synovium of RA patients.

Alternatively, IFN β might be more useful as part of combination therapy in RA. Our previous studies suggested that the concurrent modulation of kinases, such as IKK ϵ in the IFN response pathway, in combination with IFN β therapy, can enhance the anti-inflammatory response and augment potency.⁷ Those studies showed that IKK ϵ deficiency suppressed chemokine expression, and that low-dose IFN β provided synergistic benefit.⁷ This

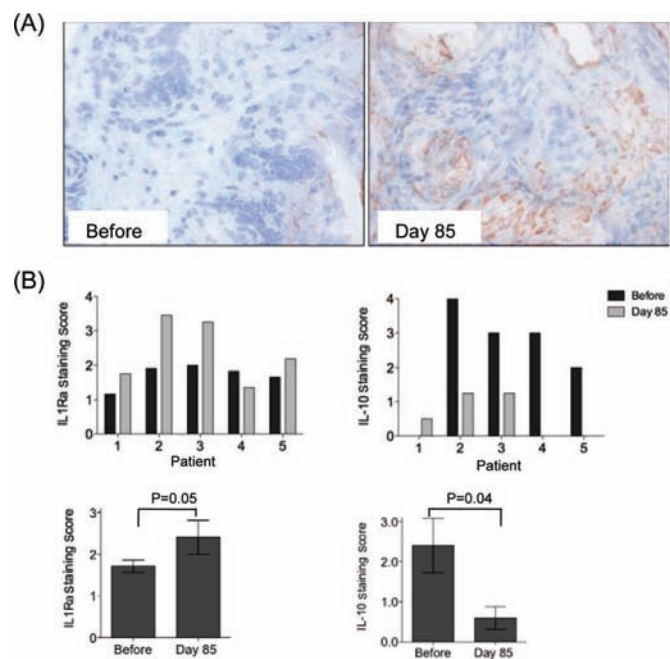


Figure 6 Immunostaining for interleukin 1 receptor antagonist (IL-1Ra) increases and interleukin 10 (IL-10) decreases after interferon (IFN) treatment in synovial biopsy specimens from rheumatoid arthritis patients. (A) Synovial biopsy specimens immunostained for IL-1Ra from the same patient before and on day 85 of IFN treatment. (B) Paired sections from blocks with biopsy specimens from five patients previously treated with IFN were immunostained with anti-IL-1Ra and IL-10. The relative staining intensity for IL-1Ra and IL-10 were visually scored on a semiquantitative scale. Shown are the scores for each patient and below are the means \pm SEM for each group. The level of IL-10 had a decreasing trend whereas the level of IL-1Ra increased ($p \leq 0.05$ by one-tailed test Mann-Whitney U test).

approach could leverage the anti-inflammatory effects of both IKK ϵ and IFN β .

In conclusion, our data suggest that IFN β acts through IL-1Ra induction for its anti-inflammatory effects in passive K/BxN arthritis. This mechanism, although effective in mice, has less utility in RA. Alternative approaches, such as local gene therapy or combination therapy with specific kinase inhibitors, could overcome these limitations.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Medical Ethics Committee of the Leiden University Medical Center.

Provenance and peer review Not commissioned; externally peer reviewed.

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